*Comp. Biochem. Physiol.* Vol. 102B, No. 4, pp. 757–760, 1992
Printed in Great Britain

0305-0491/92 $5.00 + 0.00
Pergamon Press Ltd

# SERINE PROTEINASE INHIBITOR PROFILES IN THE HEMOLYMPH OF A WIDE RANGE OF INSECT SPECIES

ANTONI POLANOWSKI,* TADEUSZ WILUSZ,* MURRAY S. BLUM,† PIERRE ESCOUBAS,†
JUSTIN O. SCHMIDT‡ and JAMES TRAVIS§‖

*Institute of Biochemistry, Wroclaw University, 50-137 Wroclaw, Poland; †Department of Entomology, University of Georgia, Athens, Georgia 30602, U.S.A.; ‡USDA-ARS, Bee Research Laboratory, Tucson, Arizona 85710, U.S.A.; and §Department of Biochemistry, University of Georgia, Athens, Georgia 30602, U.S.A. Tel.: (404) 542–1334; Fax: (404) 542-1738

(*Received* 10 *January* 1992)

**Abstract**—1. The inhibition of trypsin, chymotrypsin, neutrophil elastase and cathepsin G, and pancreatic elastase by the hemolymph of 14 insect species in six orders has been investigated.

2. All samples showed great diversity in terms of both total proteinase inhibitory capacity and specificity.

3. The highest total inhibitory capacity was found in the larval hemolymph of species in the beetle family Tenebrionidae and the lowest in that of an adult coreid bug, *Acanthocephala femorata*.

## INTRODUCTION

Proteinase inhibitors form a significant part of the total proteins in vertebrate sera (Laskowski and Kato, 1980). Extensive studies have established that the primary role of this major class of functional proteins is to control the catalytic activity of a wide diversity of proteolytic enzymes with distinct specificities which are involved in the regulation of a wide variety of metabolic processes (Heimburger, 1975; Travis and Salvesen, 1983). In invertebrate sera it has previously been demonstrated that insect hemolymph exhibits antiproteolytic activity (Law and Wells, 1989); however, studies on proteins possessing this capability have been limited to relatively few species, all of which possess a so-called complete metamorphosis (endopterygotes with internal wing development in the larval forms). Inhibitors with specificities primarily towards trypsin and/or chymotrypsin have already been isolated and characterized in larvae of the moths *Bombyx mori* (Sasaki, 1978, 1984, 1985, 1988; Sasaki and Kabayashi, 1984; Eguchi, 1982; Eguchi and Shomoto, 1984, 1985; Yoshida *et al.*, 1990), *Anticarsia gammatalis* (Boucias and Pendland, 1987) and *Manduca sexta* (Ramesh *et al.*, 1988; Kanost *et al.*, 1989; Kanost, 1990), and the fruit fly *Drosophila melanogaster* (Kangs and Fuchs, 1980). However, at this juncture there has not been a comparative examination of the number and diversity of inhibitors present in the hemolymph of species with simple or "incomplete" metamorphosis (exopterygotes with external wing development) or, for that matter, any species other than moths or flies, with complete metamorphosis. Therefore, the aim of the present study was to determine various inhibitory spectra of typical proteinase examples in the hemolymph of 14 species, including seven with complete metamorphosis and seven with "incomplete" metamorphosis, using trypsin, chymotrypsin, cathepsin G and pancreatic and leucocyte elastases.

‖To whom correspondence should be addressed.

## MATERIALS AND METHODS

The species of insects selected for this study included members of 10 families in six orders (origins in parentheses) as follows: Coleoptera—mealworm, *Tenebrio molitor* (laboratory culture) and guano beetle, *Zophobas rugipes* (laboratory culture), both Tenebrionidae and Palo Verde beetle, *Derobrachus geminatus* (Tuscan, AZ), Cerambycidae; Lepidoptera—cabbage looper, *Trichoplusia ni* (laboratory culture), and corn earnworm, *Heliothis zea* (laboratory culture), both Noctuidae and larvae and adults of the tobacco hornworm, *Manduca sexta* (laboratory culture), family Sphingidae; Hymenoptera—honey bee, *Apis mellifera* (Tucson, AZ), family Apidae; Orthoptera—eastern lubber grasshopper, *Romalea guttata* (Athens, GA), and horse lubber grasshopper, *Taeniopoda eques* (Portal, AZ) both Romaleidae, house cricket, *Acheta domesticus* (laboratory culture), family Gryllidae; Dictyoptera—American cockroach, *Periplaneta americana* (laboratory culture), family Blattidae and the Madagascar hissing cockroach, *Gromphadorhina portensa* (laboratory culture), family Blaberidae; Hemiptera—agava bug, *Acanthocephala femorata* (Tucson, AZ), and the bud coreid, *Euthochta galeator* (Beltsville, MD), both Coreidae.

Chemicals were obtained as follows: porcine trypsin, bovine chymotrypsin, porcine pancreatic elastase (PPE), *N*-benzoyl-DL-arginine *p*-nitroanilid (BAPNA), *N*-succinyl–Ala–Ala–Pro–Phe–*p*-NA, *N*-succinyl–Ala–Ala–Ala–*p*-NA and *N*-methoxy-succinyl–Ala–Ala–Pro–Val–*p*-NA, from Sigma; acrylamide, bis-acrylamide and *N,N,N,N*-tetramethylethylenediamine (TEMED) from Bio-Rad (Richmond, CA); edestin from Koch-Light Laboratories Ltd. Human leucocyte elastase (HLE) and cathepsin G (cat. G) were purified by the method of Baugh and Travis (1976).

### *Preparation of hemolymph*

Fifth-instar larvae were bled from severed prolegs, with the hemolymph being collected in a capillary pipet and transferred to an Eppendorf tube containing a few crystals of phenylthiourea to inhibit melanization. Hemolymph was

centrifuged at 10,000 *g* for 10 min at 0°C and the supernatant stored at −20°C until used. The hemolymph from adult insects was collected with a calibrated capillary pipet after cutting the anterior legs or antennae and transferred directly to two volumes of 0.2 M Tris–HCl buffer, pH 8.0. After centrifugation, this "stock" solution of hemolymph was used for all determinations.

*Proteinase activity and inhibitor measurements*

Enzyme activities were measured spectrophotometrically at 25°C in a final volume of one ml, using 1 mM chromogenic substrates as follow: BAPNA for trypsin (Erlanger *et al.*, 1961), *N*-methoxy-suc–Ala–Ala–Pro–Val–*p*-NA for HLE, *N*-suc–Ala–Ala–Pro–Phe–*p*-NA for chymotrypsin and cat. G (Nakajima *et al.*, 1979) and *N*-suc–Ala–Ala–Ala–*p*-NA for PPE (Bieth *et al.*, 1974). The following buffers were used for enzyme assays: 0.2 M Tris–HCl, pH 8.0, 5 mM CaCl for trypsin, chymotrypsin and PPE; 0.1 M phosphate buffer, pH 7.4, 0.15 M NaCl for HLE, 0.1 M Tris–HCl, pH 7.5, 0.5 M NaCl for cat. G. Enzymatic reactions were terminated by the addition of 50 μl glacial acetic acid and the released 4-nitroanilide, then measured at 405 nm.

Determination of the concentration of active enzymes was performed using the following procedure: the molar concentration of a stock solution of porcine trypsin was determined by spectrophotometric titration with NPGB (Chase and Shaw, 1970). This standardized trypsin solution was used to titrate human α-1 proteinase inhibitor (α-1 PI) which served as a secondary standard for determining the activity of other serine proteinases used in this study. Proteinase inhibitory capacity (IC) was determined by reacting constant amounts of a given enzyme with an increasing volume of hemolymph in an assay buffer for 15 min and measuring residual proteinase activity with appropriate substrate.

The number of trypsin and/or chymotrypsin inhibitors was determined after electrophoresis of hemolymph proteins in 7.5% polyacrylamide gel containing 0.1 edsestin, pH 8.6. After electrophoresis, the gels were incubated in 0.2 M Tris–HCl buffer, pH 8.0 containing 10 μg/ml of trypsin or chymotrypsin at 37°C until the visible inhibitory bands (undigested edestin) appeared. The reaction was stopped either by replacing the enzyme solution with 3% trichloroacetic acid or by washing the gel with three consecutive changes of water (30 min each) following by staining with a 0.1% solution of Amido Black 10B in a mixture of methanol–acetic acid–water (3:1:6). Gels were destained with 7% acetic acid.

*Polyacrylamide gel electrophoresis*

Electrophoresis was carried out according to Davis (1964) in tube gels (5 × 80 mm) of 7.5% acrylamide, 0.2% bisacrylamide which contained the copolymerized proteins substrate edestin. Briefly, to 5 ml of 30% acrylamide, 0.8% bisacrylamide, 6 mg of ammonium persulfite in 8 ml of water were added, followed by 2 ml of 1% edestin dissolved in 20 mM acetic acid. After 5 min incubation, 5 ml of buffer (48 ml 1 N HCl, 36.6 g Tris–base, 0.46 ml TEMED in 100 ml) were added and tube gels were cast. Samples (up to 250 μg of protein) were applied in the electrode buffer (0.6 g Tris, 2.88 g glycine in 1000 ml, pH 8.6) containing 10% sucrose and 0.001% Bromophenol Blue. Electrophoresis was carried out at room temperature at 4 mA constant current per tube. Gels were subjected to electrophoresis until the tracking dye reached the bottom.

*Protein determination*

Protein content was determined by the Coomassie dye binding method of Bradford (1976) or the bicinchoninic acid method according to Smith *et al.* (1985).

## RESULTS AND DISCUSSION

Samples of hemolymph obtained from the larvae of six species and the adults of eight species (Table 1) represented a good cross section of insect species equally divided between orders with both "incomplete" and complete metamorphosis. Protein concentration in the hemolymph varied greatly among the species ranging from 1.2% in *A. domesticus* to 10% in *T. ni*.

Titration of the hemolymph with selected proteinases showed that their inhibition increased linearly with increasing volume of hemolymph up to at least 50%. The extrapolation of the linear part of the inhibition curves yielded the number of microliters of hemolymph required for inhibition of a given amount of enzyme. This value, reflecting the antiproteolytic activity, was used to calculate the inhibitory capacity in terms of micrograms of enzyme inhibited per ml of hemolymph tested (Table 1).

*Trypsin inhibition by hemolymph*

All samples of hemolymph showed antitrypsin activity though their inhibitory capacities varied greatly among the species investigated. Utilizing this criterion they could be arbitrarily divided into groups of low (up to 100 μg/ml), moderate (110–130 μg/ml) and high (above 300 μg/ml) trypsin inhibitory capacity (TIC). Within the first group, the lowest TIC was found with *A. mellifera* (22 μg/ml), followed

Table 1. Serine proteinases inhibitory capacity of insect hemolymphs

| Species | Stage | Protein (mg/ml) | Trypsin (IC*) | Chymotrypsin (IC) | Cathepsin G (IC) | HLE (IC) | PPE (IC) |
|---|---|---|---|---|---|---|---|
| *Zophobas rugipes* | L | 68.0 | +++ | + | ++++ | ++ | ++++ |
| *Tenebrio molitor* | L | 66.0 | +++ | + | +++ | ++ | ++++ |
| *Derobrachus geminatus* | A | 27.0 | + | + | ND | ND | ++ |
| *Trichoplusia ni* | L | 100.0 | +++ | + | + | + | +++ |
| *Manduca sexta* | L | 21.0 | +++ | + | + | ++ | ++ |
| *Heliothis zea* | L | 86.0 | ++ | ++ | +++ | +++ | +++ |
| *Apis mellifera* | L | 92.0 | + | +++ | ++ | ND | + |
| *Romalea guttata* | A | 24.5 | + | ++ | ++ | + | + |
| *Taeniopoda eques* | A | 17.7 | ++ | ++ | ++ | + | ++ |
| *Acheta domesticus* | A | 12.0 | ++ | + | +++ | + | +++ |
| *Periplaneta americana* | A | 46.0 | +++ | + | ++++ | + | ++ |
| *Gromphadorhina portensa* | A | 45.0 | ++ | + | + | ++ | +++ |
| *Acanthocephala femorata* | A | 26.0 | + | + | ND | ND | + |
| *Euthochta galeator* | A | 92.0 | ++ | +++ | +++ | ND | ++ |

L: larva; A: adult.
*Inhibitory capacity: ++++ 830–1250 μg/ml, +++ 300–720 μg/ml, ++ 110–250 μg/ml, + 5–100 μg/ml, ND: not detectable.

by *D. geminatus*, *M. sexta* (adult), *A. femorata* (40–44 μg/ml) and *R. guttata*, (92 μg/ml). This group includes hemolymph from four of eight adult species sampled and only one of six larval species (Table 1). The second group included hemolymph from adults of four species: *E. galeator*, *A. domesticus*, *T. eques* and *G. portensa*, with TICs ranging from 110 to 265 μg/ml and one larval hemolymph derived from *H. zea* (130 μg/ml). The third group possessed a TIC close to, or comparable to, that of human plasma (650 μg/ml). It included hemolymph derived from an adult of one species, *P. americana* and larvae of four species: *T. ni*, *T. molitor*, *M. sexta* and *Z. rugipes* with TICS of 360, 360, 420, 440 and 560 μg/ml, respectively. It is worth noting that the hemolymph of the adult of *M. sexta* exhibited only about 8% of the larval hemolymph TIC. Since the larval hemolymph contained the lowest amount of protein of any of the species, its specific activity was much higher than that of the other species.

In order to determine the number of proteins possessing anti-trypsin activity, the hemolymph of selected species was subjected to electrophoresis in polyacrylamide containing edestin followed by exposure to trypsin. This assay revealed that, under the conditions used, the hemolymph of each species tested could be resolved into several trypsin inhibitors as follows (Table 2): *H. zea* and *R. guttata*—six bands, *A. domesticus*—four bands, *M. sexta*, *A. mellifera*, *T. eques* and *T. ni*—two bands. The hemolymph of *E. galeator* showed no inhibitor bands in spite of the fact that it possessed considerable antitrypsin activity. The reasons for this may be due to either (a) the very basic character of the inhibitor(s) which prevented entrance into the gel or (b) the possibility that this hemolymph contained a temporary trypsin inhibitor which became degraded upon incubation with trypsin.

*Chymotrypsin and cat. G inhibition*

Chymotrypsin and cat. G, despite possessing similar substrate specificities, were found to be inhibited in different manners by the hemolymphs derived from the different species tested. In general, the sensitivity of chymotrypsin to inhibition by each hemolymph was lower than that for cat. G. Chymotrypsin inhibitory capacity was found to be high only with hemolymph of *A. mellifera* (600 μg/ml) and *E. galeator* (560 μg/ml). The group of moderate inhibitory capacity comprised the hemolymph of *T. eques* (250 μg/ml), *R. guttata* (166 μg/ml), *H. zea* (160 μg/ml) and *M. sexta* (150 μg/ml). All remaining species showed rather low inhibitory capacities (below 100 μg/ml) (Table 1). Surprising results were obtained for the inhibition of cat. G. In the hemolymph of five species (*T. ni*, *M. sexta*, *G. portensa*, *A. femorata* and *D. geminatus*) the anti-cat. G activity was barely detectable, or not detectable at all. However, the hemolymph of the remaining species showed considerable cat. G inhibitory capacity. In particular, the hemolymphs of *Z. rugipes*, *P. americana* and *T. molitor* were very efficient in inhibiting this enzyme, and their capacities were calculated to be 1090, 1040 and 720 μg/ml, respectively. The only species which showed similar inhibitory capacities for both enzymes were *T. eques* and *E. galeator*. Electrophoretic determination of the number of chymotrypsin inhibitors in individual hemolymphs gave the following results (Table 2): *H. zea*—four bands; *M. sexta* and *T. ni*—five bands; *A. mellifera*—one band; *R. guttata*, *A. domesticus*, *E. galeator* and *T. eques*—three bands.

Table 2. Trypsin and chymotrypsin inhibitors in the hemolymph of insects

| Species | Stage | Trypsin inhibitors | Chymotrypsin inhibitors |
|---|---|---|---|
| *Heliothis zea* | L | 6 | 4 |
| *Manduca sexta* | L | 2 | 5 |
| *Trichoplusia ni* | L | 2 | 5 |
| *Apis mellifera* | L | 2 | 1 |
| *Romalea guttata* | A | 6 | 3 |
| *Acheta domesticus* | A | 4 | 3 |
| *Euthochta galeator* | A | 0 | 3 |
| *Taeniopoda eques* | A | 2 | 3 |

L: larva; A: adult.
The number of inhibitors was determined after separation of hemolymph proteins in PAGE containing 0.1% edestin. (See Methods for details).

*Inhibition of PPE and HLE*

HLE and PPE share similar substrate specificities but, as in the case of chymotrypsin and cat. G, their susceptibility to inhibition by insect hemolymphs varies considerably. Indeed, the large variability in inhibitory capacity among species is quite remarkable. For example, for HLE, only the hemolymph of *H. zea* had high inhibitory capacity (430 μg/ml). *T. molitor*, *Z. rugipes*, *M. sexta* and *G. portensa* showed capacities ranging from 110 to 170 μg/ml, and of the remaining ten species, five had either a very low capacity or non-detectable anti-HLE inhibitory activity (five species). On the other hand, pancreatic elastase was inhibited to various degrees by all samples of hemolymph tested. The low PPE inhibitory capacity group included four species (adult *M. sexta*, *R. guttata*, *A. femorata* and larval *A. mellifera*) with capacities of 24–77 μg/ml; the moderate group was composed of five species (*D. geminatus*, *E. galeator*, *T. eques*, *P. americana* and larval *M. sexta*) with capacities of 150–210 μg/ml; the high inhibitor capacity group included *T. ni* (310 μg/ml), *G. portensa* (340 μg/ml), *A domesticus* (360 μg/ml), *H. zea* (440 μg/ml), *T. molitor* (830 μg/ml) and *Z. rugipes* (1250 μg/ml). The inhibitor capacity of the hemolymph of the last two species towards PPE may be considered as very high and is comparable to that of the sera of both the mini pig and hamster (Schulz, 1989). *H. zea* was the only species whose hemolymph had similar inhibitory capacities towards both elastases. It is important to point out that the high elastase inhibitory capacities of the hemolymphs were positively correlated with those found for trypsin and, in some cases, for cat. G. It is also worth noting that the hemolymph from the two tenebrionid species tested contained the highest combined antiproteinase activity against trypsin, cat. G and PPE. Due to the low susceptibility of edestin to elastase digestion, the number of elastase inhibitors in hemolymphs tested could not be determined.

Although the numbers of species that we have studied is too small to generalize with, in regard to proteinase inhibitors in insect hemolymph, our data

do suggest that there is a great diversity both in the number of proteins present and in their inhibitory capacities. Furthermore, the presence of very high concentrations of specific inhibitors in the hemolymph of species with both "incomplete" (anti-cathepsin G in *P. americana*) and complete (antichymotrypsin in *A. mellifera*) metamorphoses suggests that the significance of these inhibitory proteins in invertebrates may not be inconsiderable. Finally, analyses of the concentrations of proteinase inhibitors in the *M. sexta* hemolymphs of early last-instar larvae and late last-instar larvae (prepupal) (unpublished data), as well as adults, demonstrate that these proteins undergo major quantitative changes during metamorphosis (Boucias and Pendland, 1987). Thus, they may constitute significant markers during the various stages of insect development.

*Acknowledgements*—We are grateful to J. R. Aldrich and W. R. Tschinkel for providing samples of *E. galeator* and *Z. rugipes*, respectively.

## REFERENCES

Baugh R. J. and Travis J. (1976) Human leucocyte granule elastase: Rapid isolation and characterization. *Biochemistry* **15,** 836–841.

Bieth J., Spiess B. and Wermuth C. G. (1974) The synthesis and analytical use of a highly sensitive and convenient substrate of elastase. *Biochem. Med.* **11,** 350–357.

Boucias D. G. and Pendland J. C. (1987) Detection of protease inhibitors in the hemolymph of resistant *Anticarsia gamatalis* which are inhibitory to the entomopathogenic fungus, *Nomuraea rileyi*. *Experientia* **43,** 336–339.

Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72,** 248–254.

Chase T. and Shaw E. (1970) Titration of trypsin, plasmin and trombin with *p*-nitrophenyl *p*-quanidinobenzoate HCl. *Meth. Enzym.* **19,** 20–27.

Davis B. J. (1964) Disc electrophoresis—II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121,** 404–427.

Eguchi M. (1982) Inhibition of fungal protease by hemolymph protease inhibitors of the silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae). *Appl. Ent. Zool.* **17,** 589–590.

Eguchi M. and Shomoto K. (1984) Comparison of five chymotrypsin inhibitors from the silkworm haemolymph. *Comp. Biochem. Physiol.* **78B,** 723–727.

Eguchi M. and Shomoto K. (1985) Purification and properties of a chymotrypsin inhibitor from the silkworm haemolymph. *Comp. Biochem. Physiol.* **81B,** 301–307.

Erlanger B. F., Kokowsky N. and Kohen W. (1961) The preparation and properties of two new chromogenic substrates of trypsin. *Archs Biochem. Biophys.* **95,** 271–278.

Heimburger N. (1975) Proteinase inhibitors of human plasma—their properties and control functions. In *Proteases and Biological Control* (Edited by Reich E. *et al.*), pp. 367–386. Cold Spring Harbor Laboratory, New York.

Kangs S. and Fuchs M. S. (1980) Purification and partial characterization of protease inhibitor from *Drosophila melanogaster*. *Biochim. biophys. Acta* **611,** 379–383.

Kanost M. R., Prasad S. V. and Wells M. A. (1989) Primary structure of a member of the serpin superfamily of proteinase inhibitors from insect, *Manduca sexta*. *J. biol. Chem.* **264,** 965–972.

Kanost M. R. (1990) Isolation and characterization of four serine proteinase inhibitors (serpins) from hemolymph of *Manduca sexta*. *Insect Biochem.* **20,** 141–147.

Laskowski M. J. and Kato I. (1980) Protein inhibitors of proteinases. *A. Rev. Biochem.* **49,** 593–628.

Law J. H. and Wells M. A. (1989) Insects as biochemical models. *J. biol. Chem.* **264,** 16335–16338.

Nakajima K., Powers J. C., Asche B. and Zimmerman M. (1979) Mapping the extended substrate binding site of cathepsin G and human leucocyte elastase. *J. biol. Chem.* **254,** 4027–4032.

Ramesh N., Sugumaran M. and Mole J. E. (1988) Purification and characterization of two trypsin inhibitors from the hemolymph of *Manduca sexta*. *J. biol. Chem.* **263,** 11523–11527.

Sasaki T. (1978) Chymotrypsin inhibitors from hemolymph of the silkworm, *Bombyx mori*. *J. Biochem.* **84,** 267–276.

Sasaki T. (1984) Amino acid sequence of a novel Kunitz-type chymotrypsin inhibitor from hemolymph of silkworm larvae, *Bombyx mori*. *FEBS Lett.* **168,** 227–230.

Sasaki T. (1985) The reactive site of silkworm hemolymph antichymotrypsin is located at the COOH-terminal region of the molecule. *Biochem. biophys. Res. Commun.* **132,** 320–326.

Sasaki T. (1988) Amino acid sequence of two basic chymotrypsin inhibitors from silkworm larval hemolymph. *Biol. Chem. Hoppe-Seyler* **369,** 1235–1241.

Sasaki T. and Kabayashi K. (1984) Isolation of two novel proteinase inhibitors from hemolymph of silkworm larva, *Bombyx mori*. Comparison with human serum proteinase inhibitors. *J. Biochem.* **95,** 1009–1017.

Schulz H., Mohr M. and Meyer M. (1989) Elastase and papain inhibition by serum of mammals. *Scand. J. clin. Lab. Invest.* **49,** 381–388.

Smith P. K., Krohn R. I., Hermanson G. T., Mallia A. K., Gartner F. H., Provenzano M. D., Fujimoto E. K., Goeke M. N., Olson B. J. and Klenk D. C. (1985) Measurement of protein using bicinchonic acid. *Analyt. Biochem.* **150,** 76–85.

Travis J. and Salvesen G. S. (1983) Human plasma proteinase inhibitors. *A. Rev. Biochem.* **52,** 655–709.

Yoshida S., Yamashita M., Yonehara S. and Eguchi M. (1990) Properties of fungal protease inhibitors from the integument and hemolymph of the silkworm and effect of an inhibitor on the fungal growth. *Comp. Biochem. Physiol.* **95B,** 559–564.